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Role of IRS1 and IRS2 in Modulating ErbB-induced Tumorigenesis

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14. ABSTRACT Previous studies suggested that the adapter proteins IRS1 and IRS2 may interact with ErbB1 or ErbB2 receptors. However, we could not clearly decipher a role of IRSs in ErbB2 induced tumorigenesis. We expanded our research to the IGF-IR receptor for which IRSs serve as the main adapter proteins for downstream signaling. In addition, IGF-IR is a promising target for anti-cancer therapy. Up to date, little is known about biomarkers for response to IGF-IR and subsequent sensitivity to IGF-IR therapy. Furthermore, it is important to identify a patient population who is most likely to benefit from IGF-IR therapy. We previously reported an IGF-I gene expression signature, based upon genes induced or repressed by IGF-I, which correlated with poor prognosis in breast cancer. Confirming that this signature can measure IGF activity, we found that the signature was reversed in three different models (cancer cell lines or xenografts) treated with three different anti-IGF-IR therapies. We originally reported that the IGF signature was present in triple-negative human breast cancers (TNBC), and found here that the signature is similarly present in TNBC cell lines. Especially TNBC cell lines were sensitive to an IGF-IR tyrosine kinase inhibitor (BMS-754807). Consistent with this, comparative gene expression analysis among the most resistant and sensitive cell lines identified 114 differentially expressed genes which confirmed TNBC as being sensitive. Furthermore, sensitivity to BMS-754807 significantly correlated to expression of the IGF gene signature. Supporting a role for IGF-IR signaling in TNBC, the primary human TNBC tumorgraft MC1 showed high levels of IGF-IR expression, activity, and IGF gene signature score, and showed growth inhibition following treatment with BMS-754807 as a single agent, and in combination with docetaxel tumor regression occurred until no tumor was palpable. This regression was associated with reduced proliferation, increased apoptosis, and mitotic catastrophe.				
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INTRODUCTION

Insulin receptor substrate 1 and 2 (IRS1 and IRS2) are adapter proteins that link signaling from upstream activators to multiple downstream effectors. IRSs modulate and coordinate multiple signaling cascades involved in normal growth, metabolism and survival suggesting that they may play a role in cancer. Indeed, IRSs are required for the transforming ability of many oncogenes and IRSs are elevated and hyperactive in many human tumors and high IRS1 levels are associated with poor prognosis in breast cancer. Adapter proteins have been shown to play an important role in epidermal growth factor receptor (ErbB2/Her2/Neu) amplified breast cancer. However, there is little known about the IRS interaction with ErbB2 in cancer development and progression. **Therefore, I hypothesize that ErbBs bind and phosphorylate IRSs, and that levels of IRSs will modulate ErbB-induced tumorigenesis.**

During the first year of my progress report I addressed this hypothesis by examining the role of IRS2 in ErbB2-mediated tumorigenesis in transgenic mice. We identified that IRS2 had no effect on ErbB2 induced tumorigenesis as identified by not altering branching morphogenesis, time to tumor formation, and metastatic potential. We therefore expanded our research to the IGF-IR for which IRS serve as a major adapter protein for downstream signaling.

IGF-IR is a major regulator of growth, survival, and migration and invasion. Once the ligands IGF-I or IGF-II bind to IGF-IR, it activates a cascade of downstream signaling events, the best characterized are the ERK1/2 and phosphatidylinositol-3'-kinase (PI3K) pathways (1).

IGF-IR is oncogenic, transforming NIH3T3 or mouse embryonic fibroblast (MEF) cells (2). We showed that overexpression of a constitutively active IGF-IR in the mouse mammary gland resulted in rapid mammary tumorigenesis (3), and this was then supported by a similar observation using inducible overexpression of wild-type IGF-IR (4). We subsequently showed that constitutive IGF-IR can transform immortalized mammary epithelial cells (MCF10A), and that growth of IGF-IR transformed mammary epithelial cells was blocked *in vitro* and *in vivo* by a new IGF-IR tyrosine kinase inhibitor (BMS-536924) (5). This same inhibitor was found to be active against many breast cancer cell lines, and was found to restore apical/basal polarity to MCF7 breast cancer cells grown in matrigel (6).

In breast cancer specimens, IGF-IR is detected at very high frequency and is overexpressed compared to normal breast and high serum IGF-I levels predict increased breast cancer risk (1). Numerous agents targeting IGF-IR have recently entered clinical trials, and early results are very encouraging (7). Two different classes of anti-IGF-IR inhibitors are in development: monoclonal antibodies which are highly specific for IGF-IR and cause downregulation of the receptor, and tyrosine kinase inhibitors which often cross-react and block insulin receptor (InsR) as well as IGF-IR action (7).

The significance of this research project is the realization that IRSs are not simply mitogenic and metabolic signaling elements, but that they have numerous other functions that strongly implicate them in cancer development and progression. I believe that this research project will increase our understanding of IRSs in modulating its upstream receptors in cancer development and progression, and may provide evidence and strategies for inhibiting IRSs as a therapeutic strategy in breast cancer.

BODY**1) Research and Training Accomplishments**

The Breast Center at Baylor College of Medicine (BCM) provides a unique training environment with multiple opportunities for me to grow as a young research scientist. In the past year, I have taken full advantage of these opportunities and will outline my primary accomplishments here.

- attended and presented data in a talk at the Sue and Lester Smith Breast Center Retreat (September 2009)
- attended and presented data in poster format at the Dan Duncan Cancer Center Symposium (November 2009)
- attended and presented data in poster format at the San Antonio Breast Cancer Symposium. For the work presented in this poster I received the San Antonio Breast Cancer Symposium Basic Science Scholarship (December 2010)
- presented data in a talk at the Breast Disease Seminar (February, 2010)
- submitted a first author research article entitled “High IGF-IR activity in triple-negative breast cancer correlates with sensitivity to anti-IGF-IR therapy” to Journal of Clinical Investigation (March 2010)

2) Research Project

Before presenting my findings of the past year (April 2009 – April 2010), I would first like to summarize the data from last year's progress report (covering April 2008 - April 2009) because the results directly affect the other aims of this research proposal. During the first year of the project, I have mainly focused on the role of IRS2 in ErbB2-mediated tumorigenesis in transgenic mice. I found that:

- Overexpression of IRS2 does not alter branching morphogenesis in adult ErbB2 overexpressing mice.
- Overexpression of IRS2 had no affect upon ErbB2-induced mammary tumorigenesis, with median time to tumor formation of 30.5 weeks, respectively.
- Overexpression of both oncogenes, IRS2 and ErbB2, resulted in tumors which are more differentiated than ErbB2 tumors. However, the difference in the tumor histology does not correlate with a faster tumor growth as shown previously in the Kaplan Meier survival plots.
- We observed that ErbB2 tumors exhibited higher angiogenesis compared to bigenic tumors. However, this does not correlate with a higher metastatic potential of MMTV-ErbB2 with 50% metastatic lung tumors compared to 53% in ErbB2/IRS2 bigenic mice.

The results of the first year showed that we could not clearly decipher the role of insulin receptor substrates (IRSs) in ErbB2 induced tumorigenesis *in vivo*. We submitted a revised statement of work for year 2 and 3 to expand our research project to other receptors such as IGF-IR for which IRSs serve as the main adapter proteins for downstream signaling. The new aims are:

Aim 1. Test whether IRSs are critical to ErbB receptor signaling and function

- i) Perform transient or stable knockdown of IRS1 or IRS2 levels using short-term si/shRNA in mammary cancer cells derived from MMTV-ErbB2 transgenic mice and examine effects on signaling downstream of both IGF-IR and ErbB receptors. (Months 12-15).
- ii) Perform transient or stable knockdown of IRS1 or IRS2 levels using short-term si/shRNA in MMTV-ErbB2 mammary cancer cells and examine effects on acini morphogenesis in three-dimensional culture and growth in soft agar (Months 12-16).
- iii) Determine the biological effect of IRS1 and IRS2 downregulation in the above model by examination of proliferation, apoptosis, and polarity in three-dimensional culture. (Months 14-20).

Aim 2. Examine the importance of IRS levels on response of breast cancer cells and mammary epithelial cells to IGF-IR signaling (Months 12-24)

- i) Correlate levels of IRSs with response to IGF-I and insulin in a large panel of breast cancer cell lines. (Months 12-18).
- ii) Determine the effect of loss of IRS-1 and IRS-2 on IGF-IR disrupted mammary acini formation (CD8-IGF-IR-MCF10A cells) (Months 15-20).
- iii) Test the effect of IRS knockdown on IGF-IR-mediated epithelial to mesenchymal transition and transformation (Months 18-24).

Aim 3. Identify biomarkers for response to IGF-IR and subsequent sensitivity to an IGF-IR small tyrosine kinase inhibitor (Months 18-36)

- i) Use transcriptomic profiling of a large panel of breast cancer cell lines to identify markers of sensitivity and resistance to an IGF-IR tyrosine kinase inhibitor (Months 18-24).
- ii) Test whether response of cells to the IGF-IR inhibitor is correlated with markers identified in Aim i (Months 24-30).
- iii) Examine if an IGF-IR inhibitor is effective in breast cancer xenografts identified as having an active IGF-IR pathway (Months 24-36).

The past year I have mainly worked on Specific Aim 2 and Aim 3 which is discussed in detail below.

Specific Aim 1. Test whether IRSs are critical to ErbB2 receptor signaling and function

Over the past year, I have worked hard to address our concerns and move the project forward. Many of the issues regarding crosstalk between IRSs and ErbB2 have not been resolved. My immediate goal after last years progress report was to assess whether IRS2 directly binds to ErbB2 via co-immunoprecipitation. However, co-immunoprecipitations have continued to be notoriously difficult to detect. Therefore, I have focused most of my attention this year on Aim 2 and 3 and will address this Aim in the upcoming year (April 2010 – April 2011).

Specific Aim 2. Examine the importance of IRS levels on response of breast cancer cells and mammary epithelial cells to IGF-IR signaling

- *Correlate levels of IRSs with response to IGF-I and insulin in a large panel of breast cancer cell lines*

To accomplish this aim we have recently collaborated with Dr Gordon Mills at UT MD Anderson Cancer Center using the high-throughput reverse phase protein arrays (RPPA) assay (8, 9). In this assay, up to 1000 protein lysates are printed with five serial dilutions in duplicate on a glass slide (max 12,000 spots) and then each slide probed with an antibody. More than 150 antibodies are validated for RPPA. We first started with an initial dose-finding pilot study for IGF-I and insulin. MCF7 cells were stimulated for 15mins with increasing doses of IGF-I and insulin, lysed and interrogated with over 100 antibodies by RPPA. The experiment was successful and we noted interesting differences in these signaling pathways. Figure 1 shows just a small fraction of the resultant heat map indicating changes in protein phosphorylation, and quantification showing dose response for several known markers such as MAPK, STAT5, p70s6K and IGF-IR. We chose 10nM IGF-I and 10nM insulin as the maximum dose to work with. Subsequently, this technique was used to correlate levels of IRSs with response to IGF-I and insulin in a large panel of cell lines. Therefore, we have taken 23 breast cancer cell lines, starved them overnight in SFM, and then incubated with 10nM IGF-I or 10nM insulin over a time course (0, 5min, 10min, 30min, 6hr, 24hr, 48hr). This was done in triplicate for a total of

621 lysates which were printed for RPPA analysis. Currently, bioinformatic analysis is ongoing to identify a signature of phosphorylation and protein levels altered by IGF-I or insulin across multiple cell lines.

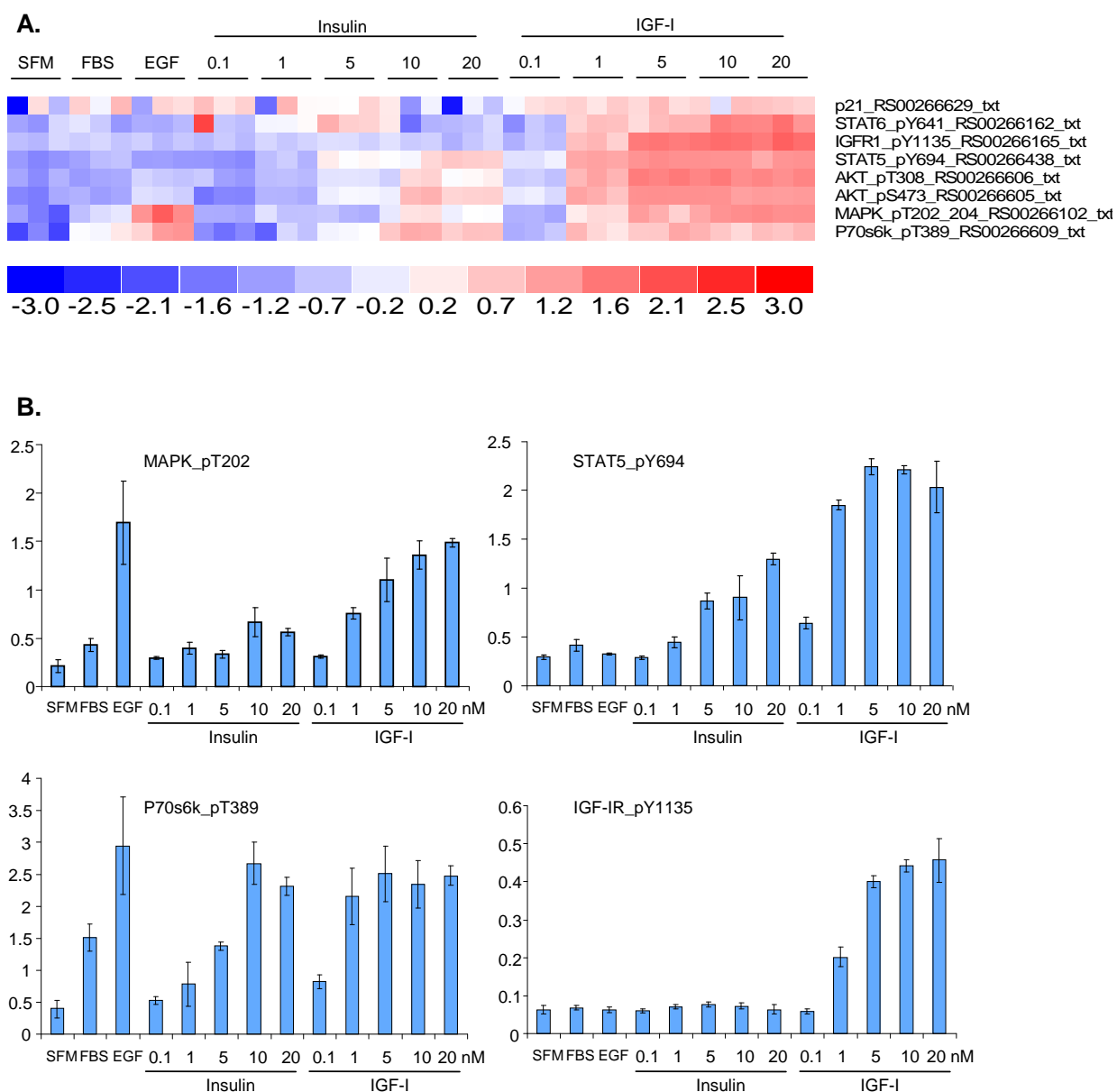


Figure 1: RPPA analysis - Initial dose-finding pilot study of MCF7 cells stimulated with insulin and IGF-I

MCF7 cells were starved overnight in SFM and then stimulated with increasing doses of insulin and IGF-I for 15mins. FBS (fetal bovine serum) and EGF (Epidermal growth factor) were used as controls. Cells were lysed and printed onto glass slides and analyzed with over 100 antibodies. **A.** Heat map showing protein phosphorylation included by insulin and IGF-I (this is a small fraction of the heat map for clarity). **B.** RPPA quantification results of the data in A presented as graphs. Each bar represents the average of biological triplicates measured in five serial dilutions in duplicate \pm S.D.

The following subaims of Specific Aim 2 have not been started.

- Determine the effect of loss of IRS-1 and IRS-2 on IGF-IR disrupted mammary acini formation (CD8-IGF-IR-MCF10A cells)
- Test the effect of IRS knockdown on IGF-IR-mediated epithelial to mesenchymal transition and transformation

Specific Aim 3. Identify biomarkers for response to IGF-IR and subsequent sensitivity to an IGF-IR small tyrosine kinase inhibitor

1. *Use transcriptomic profiling of a large panel of breast cancer cell lines to identify markers of sensitivity and resistance to an IGF-IR tyrosine kinase inhibitor*

The lack of biological effect of IRS2 on ErbB2 induced tumorigenesis in the transgenic mice lead us to expand our research project to the IGF-IR receptor for which IRSs serve as the main adapter proteins for downstream signaling. Experimental studies *in vitro* and *in vivo* have provided substantial evidence for a role of IGF-IR in human breast cancer. Overexpression of a constitutively active IGF-IR or inducible overexpression of wild-type IGF-IR in the mouse mammary gland results in rapid mammary tumorigenesis (3, 4). Consistent with this, overexpression of IGF-IR transforms immortalized mammary epithelial cells (MCF10A) (5, 10, 11). Clinical studies support the importance of IGFs in breast cancer. In breast cancer specimens, IGF-IR is detected at very high frequency and is overexpressed compared to normal breast. In addition, high serum IGF-I levels predict increased breast cancer risk (7).

To identify markers for response to IGF-IR therapy and subsequent sensitivity to an IGF-IR small tyrosine kinase inhibitor we used a biased and an unbiased approach. In the biased approach we used an 'IGF-I gene signature' pattern of genes up- or down-regulated by IGF-I that we previously reported (12). The IGF-I gene signature was derived from MCF7 cells that were starved over night and then stimulated with IGF-I for 3 hours and 24 hours. RNA was isolated and microarray analysis was performed. Only genes modulated by IGF-I at both time points were included in the IGF-I gene signature. In addition, we attempted to reduce the importance of proliferative genes by 1) removing genes annotated as being associated with proliferation in Gene Ontology (GO), and 2) removing genes that were found to be induced in fibroblasts stimulated to proliferate by serum (12). In our previous report we found that this IGF signature was present in human breast cancers, specifically the subtypes luminal B and TNBC (12). Triple-negative breast cancers (TNBC) are characterized by low to absent expression of ER, progesterone receptor (PR) and HER-2 (13, 14) and account for up to 20-25% of all breast cancers. TNBC currently has no targeted therapies, and often responds poorly to chemotherapy (15). TNBC preferentially affects younger women and African-American women, and is associated with high histological grade and aggressive clinical behavior (16).

We first set out to examine the effect of IGF-IR inhibitors on the IGF-I gene signature. We reasoned that regulation of genes by IGF-I (which made up the IGF gene signature) should be reversed by anti-IGF-IR inhibitors. Consistent with this, when we examined the levels of genes we previously found to be induced or repressed by IGF-I in neuroblastoma xenografts treated with an anti-IGF-IR (h10H5, Genentech) antibody (17), we found that there was a striking reversion in their levels (Fig. 2A). Thus, genes induced by IGF-I in MCF7 cells were repressed by h10H5 treatment of the neuroblastoma xenograft, and genes repressed by IGF-I

were now induced. This result was highly significant (Fig 2D, Spearman's rank sum $p \sim 0$). Highly similar results were obtained with an IGF-IR tyrosine kinase inhibitor (A-928605, Abbott) (18) that was administered *in vitro* to NIH3T3 fibroblasts transfected with the IGF-IR (Fig. 2B). Finally, we generated gene expression data from colon cancer xenografts (GEO) grown *in vitro* and then treated with an anti-IGF tyrosine kinase inhibitor BMS-754807 (12.5mg/kg/day) for various time-points of 1, 6 and 24 hours and 15 days. Treatment of these GEO xenografts resulted in gene expression values that were again reversed compared to the IGF-signature (Fig. 2C). Note that there was no change in the IGF signature after 1 hour, but after 6 hours of exposure to the drug a strong reversion of the IGF signature was seen, which is entirely consistent with the pharmacodynamics and pharmacokinetics of BMS-754807. **This data gives us confidence that our signature can measure IGF activity, and furthermore, suggests that the IGF signature might be a predictor of cells that have an active IGF pathway and thus may response to an IGF-IR inhibitor. Thus, the IGF-gene signature itself might be a marker of sensitivity and resistance to IGF-IR therapy.**

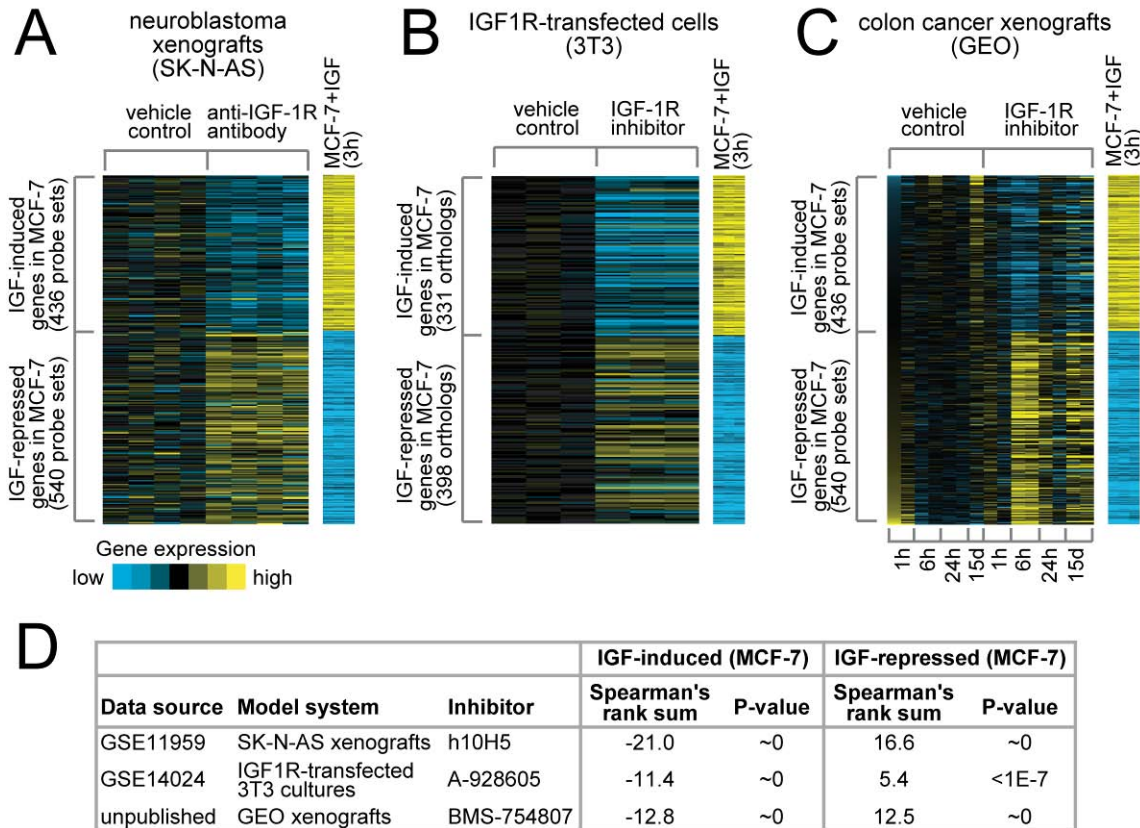


Figure 2: An IGF gene signature is reversed by treatment of cancer xenografts and IGF-IR overexpressing NIH3T3 fibroblasts with anti-IGF-IR inhibitors.

Genes in the IGF-I gene signature derived from MCF7 cells stimulated with IGF-I (12) were examined in **A**, a gene expression profile of neuroblastoma (SK-N-AS) xenografts treated with vehicle or an anti-IGF-R antibody and in **B**, IGF-IR transfected NIH3T3 fibroblasts treated with an IGF-IR small molecule inhibitor and in **C**, colon cancer xenografts treated with the small molecule inhibitor BMS-754807. For A-C, relative gene expression is represented using a yellow–blue color scale; patterns for genes that are upregulated (yellow) in the IGF gene signature are separate from the patterns for genes that are downregulated (blue). **D**, Statistical enrichment of the MCF7 IGF-I signature gene sets within each of the indicated IGF-I inhibitor treatment expression profile datasets. All genes represented in the IGF-I inhibitor dataset were first

ranked by expression in inhibitor/control (using two-sided t-test), then the overall positions of the MCF7 gene sets within the ranked lists was assessed by Spearman's rank sum (positive rank sum, associated with inhibitor group; negative rank sum, associated with control group). In all three datasets, treatment with IGF-IR inhibitors reversed expression of the IGF regulated genes.

To further identify markers of sensitivity and resistance to an IGF-IR tyrosine kinase inhibitor we used an unbiased transcriptomic approach by performing comparative gene expression analysis between sensitive and resistant breast cancer cell lines to BMS-754807 (19). Therefore, we first determined the sensitivity of BMS-754807 by MTS assay in a panel of 30 breast cancer cell lines. Among the different tumor cell lines, sensitivity presented as IC_{50} to BMS-754807 varied widely from 0.1 μ M to 25 μ M (Fig. 3). When defining cell lines as sensitive or resistant based on the median IC_{50} (6.4 μ M), we found a clear correlation between the sensitive/resistant classification to BMS-754807 and specific breast cancer subtypes. The greatest response to BMS-754807 was in basal-like/TNBC cell lines (10/15), while luminal breast cancer cell lines and Her2 overexpressing cell lines were relatively resistant (13/15) (Fig 3). Compared to all cell lines, the TNBC group was enriched for cell lines with low IC_{50} ($p < 0.01$, Spearman's rank sum, two-sided).

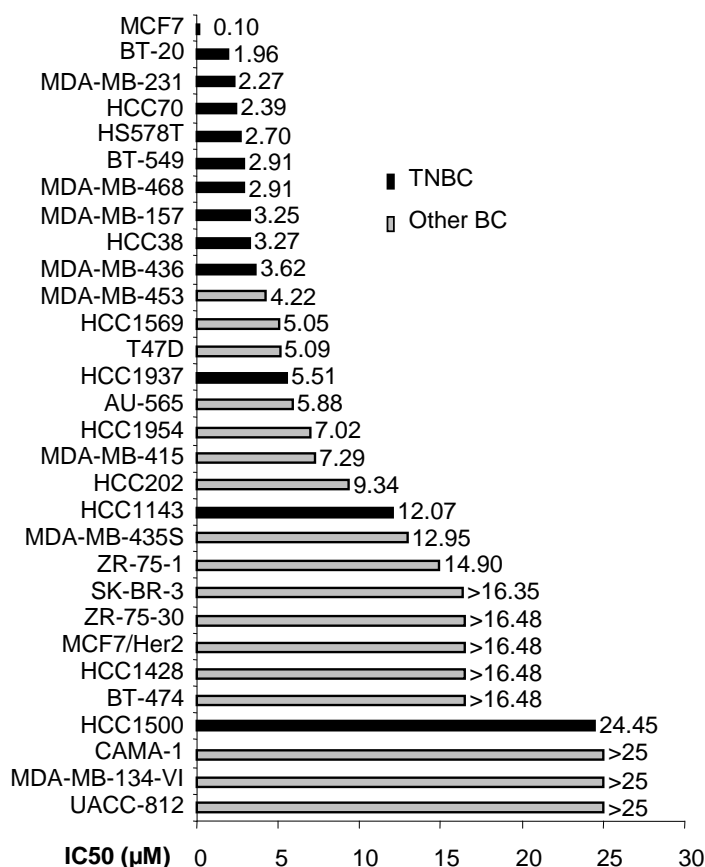


Figure 3: BMS-754807 is active in basal-like/triple-negative breast cancer cell lines. The concentration of BMS-754807 required to reduce growth by 50% (IC_{50}) was calculated for each cell line using monolayer proliferation and MTS assay. Breast cancer cells were seeded at 1,000 to 12,000 cells per well depending on the cell line in 96 well microtiter plates and incubated overnight. BMS-754807 was serially diluted and added. After 72 hr exposure, MTS assay was performed. Bars represent the average IC_{50} (μ M) of each breast cancer cell line. For seven cell lines the IC_{50} was not reached (16.49 μ M and 25 μ M, respectively). Sensitive cell lines have an IC_{50} below the mean of the group of cells (6.4 μ M); resistant cell lines are above the mean. The graph shows cell lines ranked according to its IC_{50} . Black bars represent cell lines that have a basal-like gene expression signature (TNBC) based upon the studies of Neve et al (20) and grey bars represent luminal or HER2 positive cell lines.

We then performed comparative gene expression analysis between the ten most sensitive cell lines with an IC_{50} below $4\mu M$ and the 9 most resistant cell lines with an IC_{50} above $14\mu M$ BMS-754807 using gene expression data published by Neve et al (20). This analysis identified 136 probe sets corresponding to 114 genes ($p < 0.001$ and $FDR < 5\%$) that were differentially expressed between sensitive and resistant cell lines. The top 10 differentially expressed genes were validated by qRT-PCR in a panel of seven sensitive and six resistant breast cancer cell lines (Fig. 4). **We identified that most of these 114 genes were actually markers of the basal or luminal subtype with sensitive cell lines expressing basal markers such as CAV1 and CAV2 whereas resistant cell lines expressed luminal markers such as ErbB3 and SPDEF.**

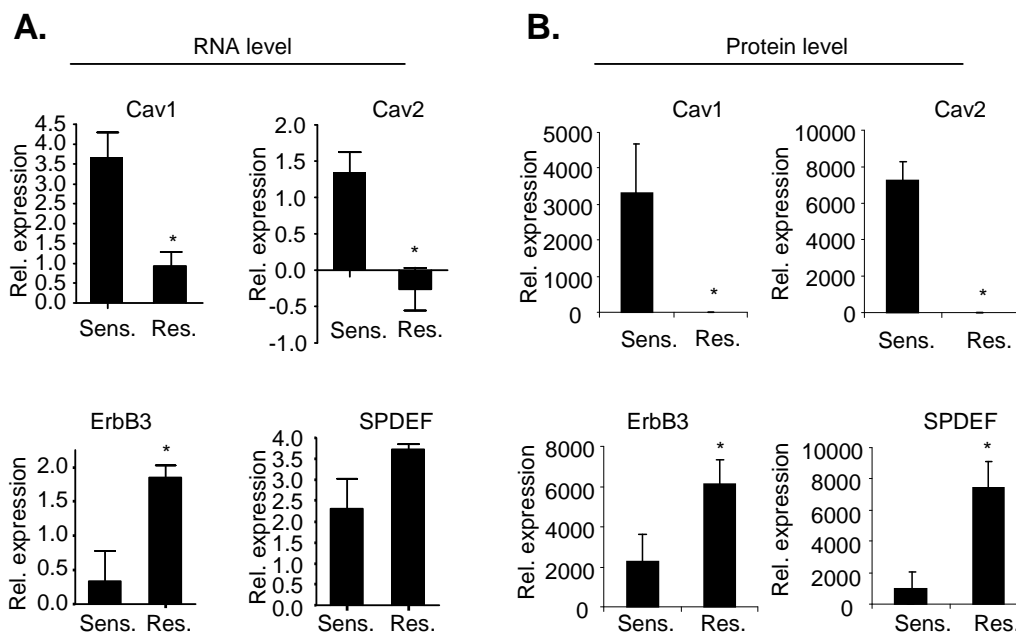


Figure 4: Sensitive cell lines express basal markers whereas resistant cell lines express luminal markers. A. The expression of differentially expressed genes were validated by qRT-PCR in a panel of seven sensitive cell lines (MCF7, BT20, MDA-MB-468, HS758T, MDA-MB-231, HCC38, MDA-MB-436) and six resistant cell lines (ZR75-1, SKBR3, BT474, CAMA-1, MDA-MB-134, UACC812). Analysis was done using the $\Delta\Delta CT$ method, normalizing first to the average of the housekeeping β -actin. The results are presented as \log_{10} transformed transcript levels. Graphs represent the mean and error bars represent the SEM. Data points were compared by the two-tailed t-test. **B.** Protein quantification values for each cell line in A were taken from Neve et al. (20). Bars represent the mean and error bars represent the SEM. Data values were compared by the two-tailed t-test.

2. Test whether response of cells to the IGF-IR inhibitor is correlated with markers identified in Aim 3.1

In the previous aim 3.1 we identified markers of response to an IGF-IR tyrosine kinase inhibitor. Based on aim 3.1 we proposed that the IGF signature might be a predictor of cells that have an active IGF pathway and thus may respond to an IGF-IR inhibitor. To test whether the presence of the IGF signature correlate with the response of cells to an IGF-IR inhibitor, we first examined the usefulness of the IGF gene signature in predicting patients who may respond to anti-IGF-IR therapy. Therefore, we studied cell lines grown in culture, where one can rapidly

assess response and analyzed the IGF-I gene signature in a publicly available dataset of gene expression profiles from a large panel of breast cancer cell lines (20). Figure 5A shows a panel of breast cancer cell lines arranged according to their intrinsic subtype (as defined using the Hoadley dataset (21)). The IGF signature is present in the majority of basal-like (and TNBC) breast cancer cell lines. We assigned each cell line a t-score based upon the similarity of its gene expression profile to the IGF signature and the positive t-score in the majority of TNBC cell lines highlights the presence of the signature in this subtype (Fig. 5B). This data is entirely consistent with our previous report of the IGF signature in TNBC (12) suggesting that the IGF-IR pathway is highly active in the triple-negative/basal-like subtype of breast cancer.

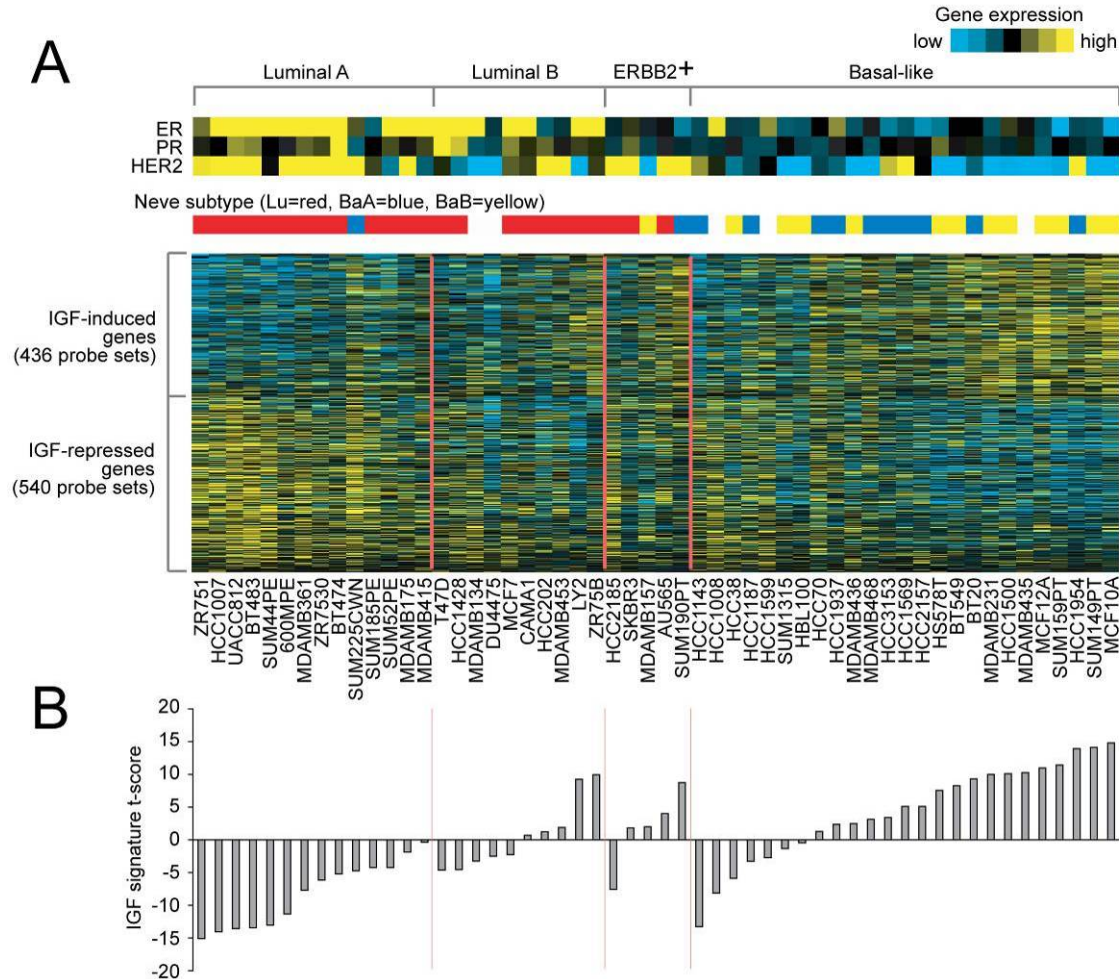


Figure 5: IGF gene signature is present in triple-negative/basal-like breast cancer cell lines. Breast cancer cell line profiles were classified using the Hoadley dataset (21), where intrinsic subtypes were previously defined. The correlation was computed between a given cell line gene expression profile and the mean centroid of each subtype; cell lines were then assigned to a subtype with the highest correlation. **A.** The heatmap represents the IGF gene signature in the cell lines according to subtype. ER, PR, and HER2 mRNA levels are shown. In addition, the breast cancer subtype is indicated as defined in the original gene expression study by Neve et al. **B.** The graph represents the t-score for each cell line based on the similarity to the IGF signature similar to previously published analyses (12). The t-score was defined as the two-sided t-statistic comparing the average of the IGF-induced genes with that of the IGF-repressed genes within each breast cancer cell line. The gene expression values in the breast cancer cell

line dataset (n=51 breast cancer cell lines, Neve et al.(20)) were first normalized to the median before computing the t-score. For each gene transcription profile dataset, we assigned intrinsic molecular subtypes to the cell lines, essentially as previously described (22), using the human tumor dataset from Hoadley et al. (21) to define the subtype-specific expression patterns.

To test whether the IGF signature is a marker of sensitivity and resistance to an IGF-IR tyrosine kinase inhibitor BMS-754807 we plotted the t-score for the IGF gene signature for each cell line against the IC_{50} (as determined in aim 3.1). There was a significant inverse correlation ($r=-0.41$, $p=0.014$), with a higher t-score (indicating an active IGF pathway) being associated with a greater response (lower IC_{50}) to BMS-754807 (Fig. 6). **These data confirm that the response of cells to BMS-754807 correlates with the IGF-gene signature. In addition, this analysis highlights that BMS-754807 is specifically active in TNBC.**

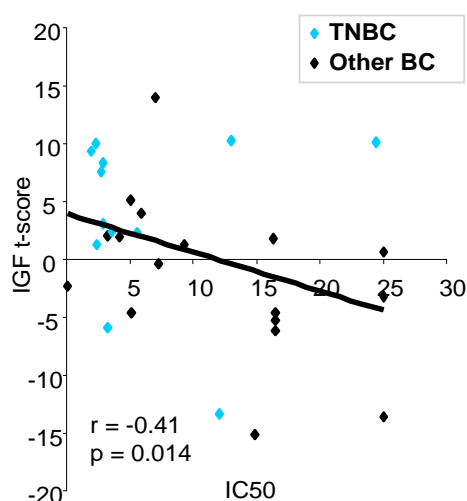


Figure 6: The IGF-I gene signature correlates with response to BMS-754807. The IC_{50} is plotted alongside the t-score for the IGF signature for each cell line. Pearson correlation shows there is a significant correlation ($r=-0.41$, $p=0.014$), with a higher t-score (indicating an active IGF-IR pathway) being associated with a greater response (lower IC_{50}) to BMS-754807. This data also highlights that BMS-754807 is active in basal-like/triple-negative breast cancer cell lines.

In the unbiased approach of aim 3.1 we identified 114 genes that were differentially expressed between sensitive and resistant cell lines. We found that most of these 114 genes are markers of the basal or luminal subtype with sensitive cell lines expressing basal markers such as CAV1 and CAV2 whereas resistant cell lines expressed luminal markers such as ErbB3 and SPDEF. To proof that these markers correlate to the response of cells to the IGF-IR inhibitor BMS-754807 we next analyzed the 114 genes in a panel of 51 breast cancer cell line profiles with known and unknown IC_{50} to BMS-754807 (20). Hierarchical clustering separated the cell lines into two major bins: ER-negative cell lines that were sensitive and ER-positive cell lines that were mostly resistant (Fig. 7).

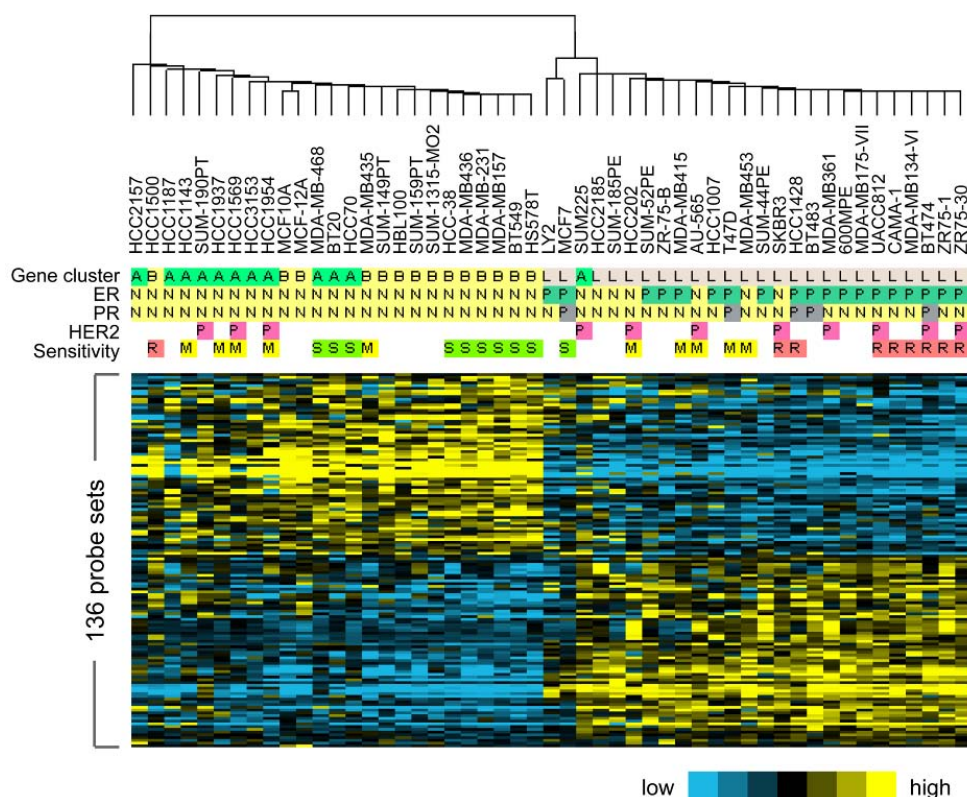


Figure 7. 114 differentially expressed genes identify triple negative breast cancer cell lines as most responsive to BMS-754807. 136 differentially expressed probe sets, representing 114 genes, were analyzed in 51 breast cancer cell line profiles published by Neve *et al.* with known and unknown IC_{50} . S=sensitive ($IC_{50}<4\mu M$), M=Medium IC_{50} : ($4\mu M<14\mu M$), R=Resistant ($IC_{50}>14\mu M$). In addition, the ER, PR and Her2 status is indicated (P = positive, N = negative). Gene-cluster indicates the breast cancer subtype as defined in the original gene expression study by Neve *et al.* (A = basal A, B = basal B, L = Luminal).

To validate this clustering data, we determined the sensitivity to BMS-754807 of breast cancer cell lines with an unknown IC_{50} by MTS-assay. Among the cell lines tested the TNBC cell lines SUM149PT and MCF10A showed the greatest response to BMS-754807 whereas cell lines that are ER negative but overexpress Her2 (SUM225 and SUM190PT) are less sensitive to BMS-754807 (Fig. 8 and Table 1). Luminal breast cancer cell lines such as ZR75B, MDA-MB-175VII and MDA-MB-316 showed the least response to BMS-754807. **In summary, these experiments showed that 114 genes are markers that correlate with response of cells to the IGF-IR inhibitor BMS-754807. In addition, we could confirm that BMS-754807 is especially active in TNBC cell lines.**

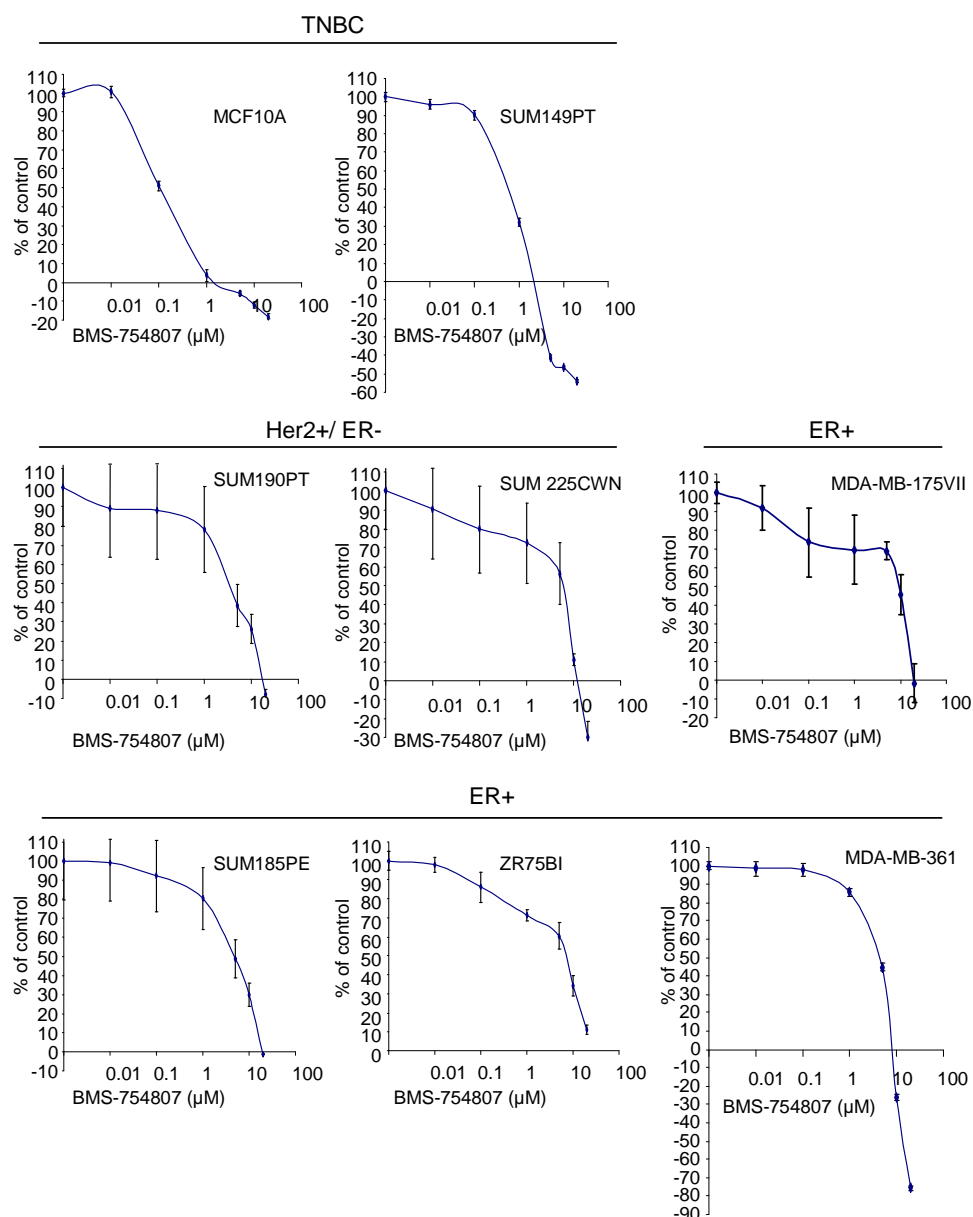


Figure 8. BMS-754807 is especially active in TNBC cell lines

Breast cancer cells with unknown IC_{50} s to BMS-754807 were plated in 96 well microtiter plates and incubated overnight. BMS-754807 was serially diluted and added. After 72 hr exposure, MTS assay was performed. Sensitive cell lines have an $IC_{50} < 4\mu M$, moderate IC_{50} between $4\mu M$ and $14\mu M$, resistant: $IC_{50} > 14\mu M$

Table 1. Summary of the effect of BMS-754807 on cells lines with an unknown IC_{50}

Cell lines	Response to BMS-754807	ER status	Breast Cancer subtype
SUM149PT	sensitive	-	TN
MCF10A	Sensitive	-	TN
SUM225CWN	Moderate	-	(HER2+)
Sum190PT	Moderate	-	(HER2+)
SUM185PE	Moderate	Low +	Luminal
ZR75B	Moderate	+	Luminal
MDA-MB-175VII	Moderate	+	Luminal
MDA-MB-361	Moderate	+	Luminal

3. Examine if an IGF-IR inhibitor is effective in breast cancer xenografts identified as having an active IGF-IR pathway

Preclinical cancer research has relied heavily upon cell lines grown in culture and then xenografted for growth in mice. However, recent work has shown that human cancers placed directly into the mouse (tumorgrafts) maybe a more appropriate model that is better at predicting response to drugs in humans (23). We have recently developed several new tumorgraft models of human TNBC. We screened seven TNBC tumorgrafts for activity of the IGF-IR by both immunohistochemistry (IHC) and immunoblotting (IB). Figure 9A shows three representative tumorgrafts expressed various levels of IGF-IR and had divergent levels of active phosphorylated pY-IGF-IR. We generated gene expression data from all of the tumorgrafts and calculated an IGF signature t-score for each tumorgraft. Analysis of these profiles showed that tumorgraft MC1 (24) had the highest level of IGF-IR and pY-IGF-IR, and in addition had a high IGF signature t-score. Protein lysates from the same tumorgrafts as in Fig 9A confirmed that MC1 had the highest activation of IGF-IR (Fig. 9B). Activation of downstream signaling molecules varied among the tumorgraft models. MC1 had high levels of IRS1 and activated AKT whereas the tumorgraft 2665A showed activation of MAPK.

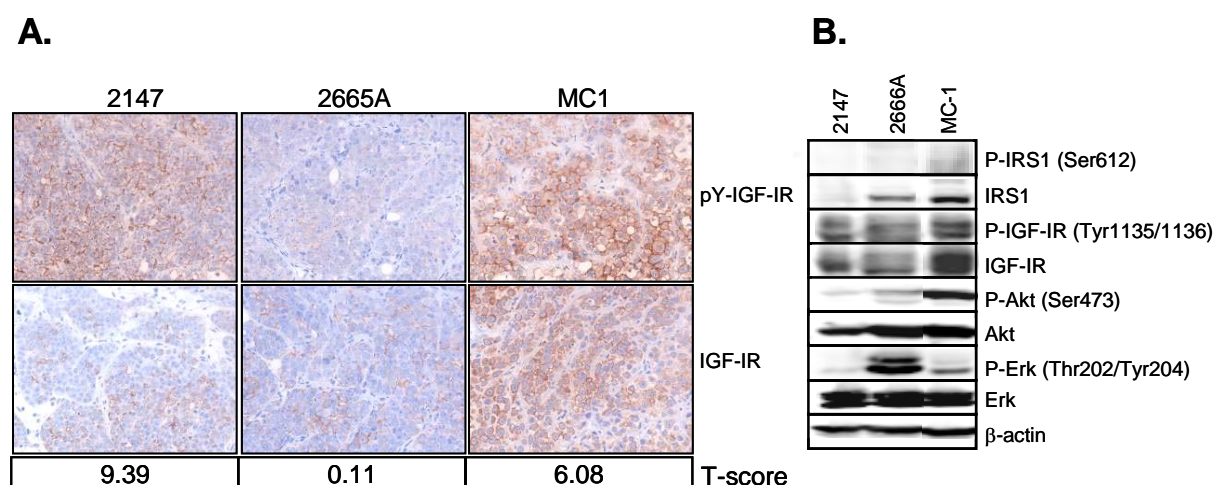


Figure 9. TNBC human tumorgrafts express high levels of IGF-IR

A. Three different TNBC human tumorgraft lines (2147, 2665A, MC1) were harvested and processed in paraffin. Immunohistochemistry was performed for anti-pY-IGF-IR (Phospho-IGF-IR) and total IGF-IR. Microarray analysis was performed on these tumorgrafts. Based on the gene expression data an IGF signature t-score was calculated for each tumorgraft as indicated underneath the representative picture. **B.** The same three tumorgraft lines were lysed and analyzed by immunoblot and probed using IGF-IR specific antibodies as well as total/phospho-specific antibodies for IRS1, AKT, ERK1/2. β-actin was used as a loading control. Tumorgraft lines that were not relevant to this study were cropped out between 2665A and MC1.

Our studies in Aim 3.1 and 3.2 identified TNBC cell lines as having an active IGF pathway. As we previously showed that BMS-754807 is most active in TNBC cell lines *in vitro* (Aim 3.1 and 3.2), we directly examined the effectiveness of the IGF-IR inhibitor BMS-754807 in the MC1 tumorgraft model of TNBC breast cancer alone as a single agent or in the presence of chemotherapy (docetaxel). We chose the tumorgraft MC1 for this preclinical study as it showed the highest levels of active and total IGF-IR and also a high IGF t-score (Figure 9). A recent

study examining timing of anti-IGF-IR therapy and chemotherapy in cells in culture showed that most efficacious combination was chemotherapy followed by anti-IGF-IR therapy (25). We thus administered docetaxel followed by BMS-754807 the next day. Single agent BMS-754807 achieved a statistically significant ($p<0.001$) reduction in tumor growth when compared with the control group (Fig. 10A). Docetaxel stabilized MC1 tumor growth. Strikingly, combined treatment with BMS-754807 and docetaxel showed superior tumor growth inhibition to either single agent alone ($p<0.001$), and four out of six mice receiving the combined agents had tumors regress until no tumor was palpable.

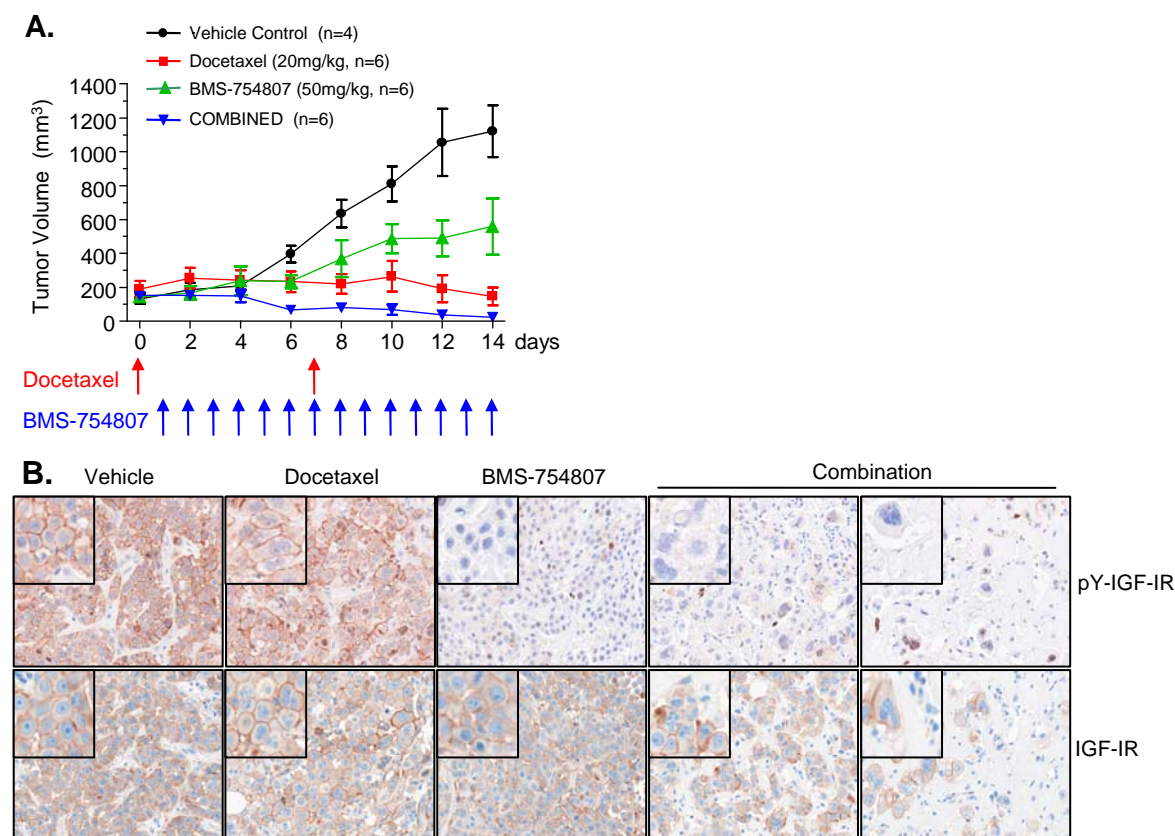


Figure 10. BMS-754807 inhibits growth of TNBC tumorgrafts and causes regression in combination with chemotherapy

A. 4 to 6-week-old female NOD/scid mice were transplanted with a 1mm³ piece of tumorgraft into a cleared number four mammary fat pad. When tumors reached a volume between 100-200mm³ they were randomized to receive the following treatments: vehicle, 50mg/kg BMS-754807 daily by oral gavage, 20mg/kg Docetaxel weekly by intraperitoneal injection or the combination of 50mg/kg BMS-754807 daily and 20mg/kg docetaxel weekly by intraperitoneal injection. Tumor volume and body weight was measured daily. All mice were sacrificed when control tumors reached 1000mm³. **B.** Untreated and treated tumors were processed in a tissue microarray (TMA) for immunohistochemical analysis. Immunohistochemistry was performed for Phospho and total IGF-IR. Representative IHC staining of the treatment groups is taken at 40x magnification.

To confirm the ability of BMS-754807 to inhibit IGF-IR activity in triple negative breast cancer, tyrosine phosphorylation and total level of IGF-IR was examined in the various treatment groups. Both p-IGF-IR and IGF-IR showed membrane staining with a small amount of cytoplasmic staining (Fig. 10B). There was no change in the levels of pY-IGF-IR between tumors treated with docetaxel or vehicle (Fig. 10B). In contrast, BMS-754807 completely blocked IGF-IR phosphorylation. There was no change in levels of total IGF-IR between the different treatment groups.

We next analyzed the histology of the tumors among the different treatment groups. Several reports have shown that chemotherapy agents such as docetaxel affect the stability of the microtubules and in doing so induce mitotic catastrophe (26, 27). Mitotic catastrophe results from aberrant mitosis, or missegregation of chromosomes followed by cell division which results in the formation of multinucleated giant cells leading to cell death. Cell death through mitotic catastrophe may occur through apoptosis as well as necrosis (27). MC1 tumorgrafts treated with Docetaxel showed relatively few multinucleated giant cells (Fig. 11A). However, the addition of BMS-754807 dramatically increased docetaxel-induced mitotic catastrophe (Fig. 11A). Analysis of H&E staining showed that normal breast cancer cells were still present in Docetaxel treated tumors whereas only multinucleated cells were present in the combination treated tumors.

Since multinucleated cells may be temporarily viable and mitotic catastrophe may be a process leading to death (27), we analyzed treated tumors for replication, proliferation, and apoptosis. All treatment groups showed a reduction in replication as indicated by less BrdU incorporation into DNA compared to untreated tumor cells (Fig. 11B). BMS-754807 alone reduced replication by 36% (22% BrdU positive cells in vehicle tumors versus 16% in treated tumors) but this was not significant (adjusted p-value=0.13). Chemotherapy significantly reduced replication by 55% (adjusted p-value=0.015) and the combination by 59% compared to vehicle (adjusted p-value=0.0018). We next analyzed proliferation as assessed by Ki67 positive cells (Fig. 11C and E). Whereas both single agents alone were able to significantly reduce proliferation by 30-32% (adjusted p-values < 0.0001), combination treatment resulted in only a 17% reduction in proliferation (adjusted p-value < 0.005, compared to vehicle). We then investigated whether single agents alone or the combination of BMS-754807 and Docetaxel induces cell death. BMS-754807 caused a 4-fold elevation in apoptosis as measured by cleaved caspase 3 (CC3) from 3% apoptotic cells in vehicle treated tumors to 11% in BMS-754807 treated tumors (Fig. 11D and E), however this elevation was not significant (adjusted p-value = 0.36). Chemotherapy resulted in a 6.7-fold induction of apoptosis compared to vehicle (adjusted p-value=0.1) which again wasn't significant, whereas the combination caused a striking 12-fold induction of apoptosis (Fig. 11D and E) which was highly significant (adjusted p-value=0.005). In addition, combination therapy resulted in massive cell destruction through necrosis as shown in Figure 11A.

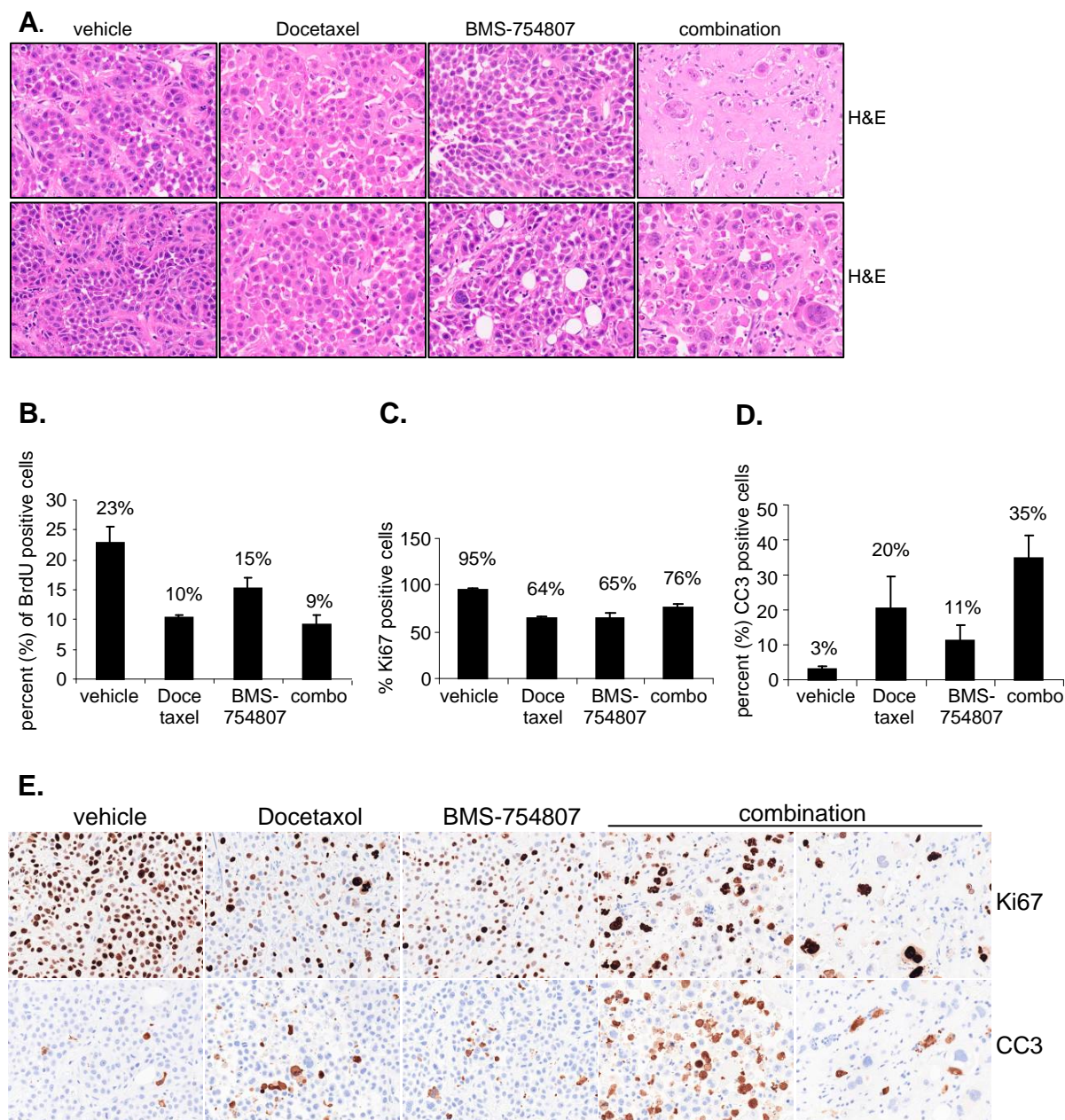


Figure 11. BMS-754807 sensitizes MC1 tumorgrafts to docetaxel induced mitotic catastrophe

A. Representative tumor sections of the treatment groups stained with hematoxylin and eosin are taken at 40x magnification. **B.** Quantification of BrdU incorporation per cell was done by image analysis. Data represents means \pm SE of 12 representative pictures per treatment group. **C and D.** The percentage of Ki67/CC3-positive cells within the tumor was scored. Values represent the means \pm SE of 12 representative pictures per treatment group. **E.** Representative tumor sections of the treatment groups stained with Ki67 and CC3 (cleaved caspase 3) are taken at 40x magnification.

KEY RESEARCH ACCOMPLISHMENTS

- The IGF-gene signature can measure IGF activity, as based on reversion of the signature in three different cancer models treated with different anti-IGF-IR therapies. Furthermore, this suggests that the IGF signature might be a predictor of cells that have an active IGF pathway and thus may response to an IGF-IR inhibitor.
- The IGF-IR tyrosine kinase inhibitor BMS-754807 inhibits proliferation in a panel of breast cancer cell lines with selective activity in triple negative breast cancer cell lines.
- Comparative gene expression analysis among the most sensitive and resistant cell lines to BMS-754807 identified 114 genes which confirmed TNBC as being sensitive to BMS-754807.
- The IGF signature is present in triple-negative/basal-like breast cancer cell lines.
- Sensitivity of cells to BMS-754807 correlates with the IGF-gene signature which highlights that BMS-754807 is especially active in TNBC cell lines.
- Finally, new tumorgraft models of TNBC show expression and activation of IGF-IR and treatment with BMS-754807 in combination with chemotherapy results in complete tumor regression.

REPORTABLE OUTCOMES

- Beate C. Litzenburger, Chad J. Creighton, Anna Tsimelzon, Bonita T. Chan, Susan G. Hilsenbeck, Fei Huang, Joan M. Carboni, Marco M. Gottardis, Jenny C. Chang, Michael T. Lewis, and Adrian V. Lee. High IGF-IR activity in triple-negative breast cancer cell lines correlates with sensitivity to IGF-IR inhibitor BMS-754807. (32nd Annual CTBC-AACR San Antonio Breast Cancer Symposium, #1132, 2009, SABCS Basic Science Scholarship)
- Beate C. Litzenburger, Chad J. Creighton, Anna Tsimelzon, Bonita T. Chan, Susan G. Hilsenbeck, Fei Huang, Joan M. Carboni, Marco M. Gottardis, Jenny C. Chang, Michael T. Lewis, and Adrian V. Lee. High IGF-IR activity in triple-negative breast cancer cell lines correlates with sensitivity to IGF-IR inhibitor BMS-754807. (Dan Duncan Cancer Center Symposium, 2009)
- Litzenburger BC, Creighton CJ, Chan TB, Tsimelzon A., Hilsenbeck SG, Carboni JM, Gottardis MM, Chang JC, Lewis MT, Lee AV. High IGF-IR activity in triple-negative breast cancer correlates with sensitivity to anti-IGF-IR therapy. (Journal of Clinical Investigations, submitted)

CONCLUSION

During the first year of this research project (April 2008-April 2009), I have mainly focused on the role of IRS2 in ErbB2-mediated tumorigenesis in transgenic mice. However, we could not clearly decipher the role of insulin receptor substrates (IRSs) in ErbB2 induced tumorigenesis *in vivo*. Therefore, we expand our research project to other receptors such as IGF-IR for which IRSs serve as the main adapter proteins for downstream signaling. During this year (April 2009 – April 2010), I have successfully worked on Aim 3 to identify biomarkers for response to IGF-IR and subsequent sensitivity to an IGF-IR small tyrosine kinase inhibitor. Originally, we developed the IGF signature to learn more about transcriptional events downstream of IGF-IR, and to examine the role of IGF-IR regulated genes in breast cancer. However, the obvious question as to the potential role of the IGF signature in predicting response to anti-IGF-IR therapy arose. We showed here that the IGF signature can measure IGF activity in tumors and that the IGF signature weakly correlated ($r=0.41$) with response of cells to an IGF-IR inhibitor. The strength of the signature may come when used in combination with IGF-IR protein levels and activity as potential marker for response to IGF-IR therapy. Therefore, IGF-IR alone, or the IGF signature alone maybe insufficient to indicate an active IGF pathway, but the combination may yield better prediction. To this end, we used this strategy to select a TNBC tumorgraft for study and found dramatic effects of an IGF-IR inhibitor. Further comprehensive studies are required to definitively prove whether the combination of IGF-IR levels and downstream gene transcripts is a useful method for identify patients who may respond to anti-IGF-IR therapy.

In addition, I started working on Aim 2 to correlate levels of IRS with response to IGF-I and insulin in a large panel of breast cancer cell lines. I believe that the research I will perform over the next few months will be extremely informative and exciting to elucidate the importance of IRS levels on response of breast cancer cells to IGF-IR signaling.

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APPENDIX

BIOGRAPHICAL SKETCH

NAME		POSITION TITLE	
Beate Litzenburger		Predoctoral Fellow	
eRA COMMONS USER NAME			
LITZENBU			
EDUCATION/TRAINING (<i>Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.</i>)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
RWTH Aachen, Aachen, Germany	M.Sc.	2000-2006	Molecular and Cellular Biology
Baylor College of Medicine, Houston, TX	Ph.D. (in progress)	2006-now	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE:

12/2006-present	Baylor College of Medicine, Houston, TX, USA Lester and Sue Smith Breast Center, Predoctoral fellow PhD Thesis
10/2005-07/2006	Baylor College of Medicine, Houston, TX, USA Lester and Sue Smith Breast Center, Master's student Master Thesis: Characterization of a small molecule inhibitor in the treatment of breast cancer
06/2005-09/2005	Grunenthal GmbH, Aachen, Germany Research & Development, Intern Project: Planning and monitoring of a clinical phase II study
01/2004-06/2004	Baylor College of Medicine, Houston, TX, USA Department of Molecular and Cellular Biology, Intern RNA-interference as a gene therapy strategy for the treatment of Epidermolysis Bullosa Simplex
2003/2004	Medical Center 'Blondelst.' Aachen, Germany Assistant Medical Technician Analysis of human serum of HIV patients using Flow Cytometry

HONORS AND AWARDS

- 2009 San Antonio Breast Cancer Symposium Basic Science Scholarship
- 2008-present Department of Defense Predoctoral Traineeship Award
- 2007-2008 Fellowship of the German Academic Exchange Service (DAAD)
- 2007 1st place oral presentation award winner at the Breast Center Retreat, Baylor College of Medicine, Houston, TX
- 2006 1st place poster award winner at the Breast Center Retreat, Baylor College of Medicine, Houston, TX
- 1999-2000 President's Honor Roll. Butler County Community College, Kansas, USA

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Litzenburger BC, Creighton CJ, Chan TB, Tsimelzon A., Hilsenbeck SG, Carboni JM, Gottardis MM, Chang JC, Lewis MT, Lee AV. High IGF-IR activity in triple-negative breast cancer correlates with sensitivity to anti-IGF-IR therapy. (Journal of Clinical Investigations, submitted)

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POSTER PRESENTATIONS

Beate C. Litzenburger, Chad J. Creighton, Anna Tsimelzon, Bonita T. Chan, Susan G. Hilsenbeck, Fei Huang, Joan M. Carboni, Marco M. Gottardis, Jenny C. Chang, Michael T. Lewis, and Adrian V. Lee. High IGF-IR activity in triple-negative breast cancer cell lines correlates with sensitivity to IGF-IR inhibitor BMS-754807. (32nd Annual CTRC-AACR San Antonio Breast Cancer Symposium, #1132, 2009, SABCS Basic Science Scholarship)

Beate C. Litzenburger, Chad J. Creighton, Anna Tsimelzon, Bonita T. Chan, Susan G. Hilsenbeck, Fei Huang, Joan M. Carboni, Marco M. Gottardis, Jenny C. Chang, Michael T. Lewis, and Adrian V. Lee. High IGF-IR activity in triple-negative breast cancer cell lines correlates with sensitivity to IGF-IR inhibitor BMS-754807. (Dan Duncan Cancer Center Symposium, 2009)

Litzenburger B.C., Michael J. Toneff, Robert K. Dearth, Hyun-Jung Kim, Isere Kuitse, Ora Britton, Yi Li, Adrian V. Lee. Constitutive activation of the insulin-like growth factor receptor accelerates ErbB2-induced mammary tumorigenesis (Gordon Research Conference, Insulin-like growth factors in physiology and disease, 2009)

Litzenburger B.C., Kim H.J., Carboni J, Fairchild C.R., Gottardis M.M., Wong T.W., Attar R.M., Lee A.V. IGF-IR inhibitor BMS-536924 causes growth inhibition and polarization of MCF7 breast cancer cells in 3D culture (Department of Medicine Research Symposium, 2008)

Litzenburger B.C., Kim H.J., Carboni J, Gottardis M.M., Wong T.W., Attar R.M., Cui X., Lee A.V. Small molecule inhibitor BMS-536924 completely reverses IGF-IR-mediated transformation of immortalized mammary epithelial cells (Gordon Research Conference, Insulin-like growth factors in physiology and disease, 2007)

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